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14. ABSTRACT The hypothesis addressed by this project is that early life exposure to stress or glucocorticoids produces a distinct neurochemical and behavioral phenotype characterized by life-long vulnerability to stressors that trigger PTSD. During the past year we completed studies on our prenatal stress model (PNS). We found that PNS programs a sensitization to conditioned fear in the adult offspring, and a resistance to the extinction of conditioned fear, but only after a prolonged adults stress. We also found that PNS programs a unique adult neurochemical and hormonal phenotype that suggests possible mechanisms by which it can increase vulnerability to traumatic stress during adulthood. A manuscript describing these results was published online June 22, 2011. Also during the past year, we have begun work on a second model, to determine whether glucocorticoid receptor stimulation is necessary and sufficient for the behavioral and neurochemical phenotype produced by PNS. We began studies to determine the effects of administration of the endogenous glucocorticoid, corticosterone (CORT), on the behavioral and neurochemical phenotypes produced by PNS. We determined a dose of corticosterone that reproduces the levels of CORT in dams and pups during prenatal stress demonstrated that the glucocorticoid antagonist, metyrapone, (MET) blocks the elevation of CORT. In an initial study, we found that prenatal CORT did not reproduce the behavioral effects of PNS, possibly due to use of DMSO as a vehicle. Prenatal CORT reproduced selected neurochemical effects of PNS, i.e. reduction of glucocorticoid receptors in the adult prefrontal cortex. However, unlike PNS, it did not reduce TH mRNA in the brainstem region containing the locus ceruleus. We have begun a second set of studies to increase the N and measure the effects of prenatal CORT on adult vulnerability to stress.				
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## Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>9</b>
<b>Reportable Outcomes.....</b>	<b>10</b>
<b>Conclusion.....</b>	<b>10</b>
<b>References.....</b>	<b>n/a</b>
<b>Appendices.....</b>	<b>11</b>
<b>Supporting Data.....</b>	<b>n/a</b>

## **A. INTRODUCTION:**

Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants which have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, we proposed the following specific aims: 1. To generate and characterize models of early life stress: prenatal stress; prenatal glucocorticoid receptor stimulation; and perinatal stress and perinatal glucocorticoid exposure. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from models developed in Specific Aim 1 are exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatments with the SSRI sertraline in adults exposed to early life stress.

## **B. BODY:**

During the last funding period (September 1, 2010 to September 28, 2011), we completed the experiments on Model 1 (prenatal stress) to address Task 1 - Determine adult predictors of vulnerability to stress; and Task 2 - Determine adult vulnerability to stress. The results of these studies were published in the journal Neuroscience and appeared in the online version on June 22, 2011.

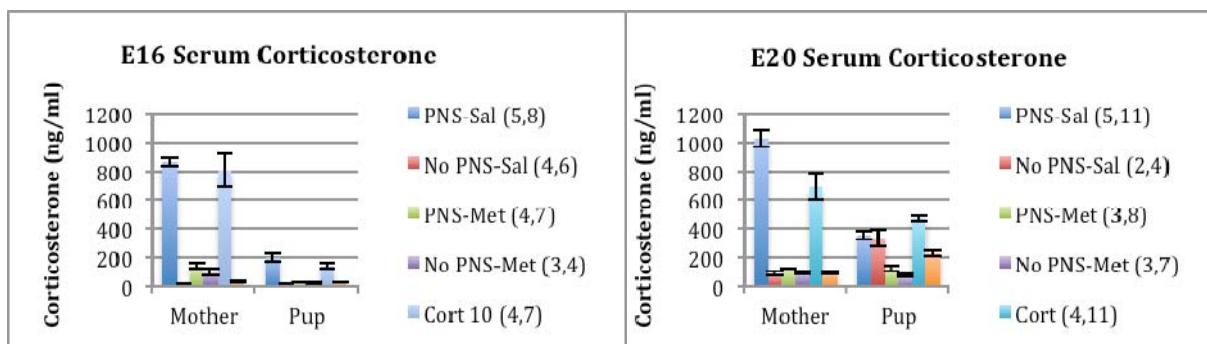
This report covers progress to date and the initiation of experiments in Year 3 to begin testing of the second of four models of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We are testing Model 3 before Model 2, because it more logically follows from the studies employing Model 1 (the effects of maternal restraint stress on adult predictors and adult vulnerability to traumatic stress). We made a minor modification to the approach in which, instead of testing dexamethasone (a synthetic glucocorticoid) in utero, we will administer the natural glucocorticoid agonist corticosterone. We have also begun another model in which we will measure the effect of the corticosteroid antagonist metyrapone administered during prenatal stress to determine the role of glucocorticoids in the effects of prenatal stress on subsequent adult changes in neurochemistry and behavior in the offspring. For Model 3, we decided against using the synthetic glucocorticoid receptor agonist dexamethasone to determine if it would mimic the effects of stress, because it is a pure glucocorticoid receptor agonist whereas the endogenous glucocorticoid (corticosterone) is an agonist at both glucocorticoid and mineralocorticoid receptors. Therefore, the natural glucocorticoid more closely mimics the effects of stress.

Moreover, by using the antagonist metyrapone and the agonist corticosterone, we will be able to determine unambiguously whether corticosterone is involved in the effects of maternal stress. In order to design an intervention, we need to know whether the naturally occurring corticosterone is involved in the effects of maternal stress, as opposed to other stress-responsive hormones such as catecholamines.

## Results:

Initial studies (Model 3, Task 1, Steps 1-4) were done to establish a range of effective doses of CORT and metyrapone which could then be tested in the pregnant females. These doses were initially established using DMSO as a vehicle for both drugs. However due to several concerns, including potential toxicity of DMSO, pup loss, stress caused by the strong odor emitted by DMSO-treated animals, and later observations of behavioral inconsistencies, the vehicles were changed to saline (for metyrapone) and sesame oil (for CORT), and the doses of metyrapone and CORT were re-established. For CORT, groups of time-pregnant SD rats were injected once daily with a range of doses (5-30 mg/kg, s.c) starting at embryonic day E14. A positive control PNS-treated group was injected with vehicle and immobilized 1hr/day. Dams were sacrificed by decapitation on day E16 or E20, and maternal trunk blood was collected. Fetuses were then rapidly removed (<5 min) and trunk blood collected with heparinized capillary tubes. Serum CORT was measured by RIA.

To establish a dose of metyrapone that blocks the stress-induced elevation in endogenous CORT by PNS exposure, pregnant rats were treated with metyrapone (50-100 mg/kg s.c.) 1 hr. prior to immobilization each day. A second PNS group was injected with vehicle 1 hr. before immobilization. Results for CORT (10 mg/kg in sesame oil) and Metyrapone (100mg/kg in saline) are shown in **Figure 1**. At both E16 and E20, PNS and CORT (10 mg/kg) induced comparable CORT increases in both maternal and fetal serum. Further, PNS-induced CORT release was effectively blocked in both mothers and fetuses by pretreatment with metyrapone. These data indicate that over the course of daily PNS, rat fetuses are exposed to a level of CORT that can be mimicked by 10 mg/kg exogenous CORT given daily, and blocked with 100 mg/kg metyrapone pretreatment.



**Figure 1: Serum CORT following treatment with PNS, metyrapone, or CORT**

Treatment was started at embryonic day E14 and measured at day E16 (left) or E20 (right). Data are expressed at the mean ± SEM for the number of animals in parentheses (mothers, pups).

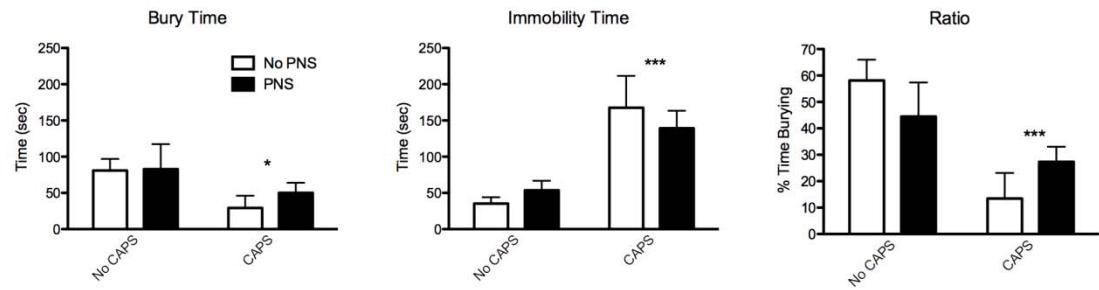
Some pups in each treatment group were tested on behavioral and molecular biology tests as adults (Model 3, Tasks 1 and 2, Steps 4 and 5).

**Behavioral Testing:** Due to the very long time between prenatal treatment and adult testing, we initiated pilot studies investigating the potential role of prenatal CORT on the adult behavioral effects of PNS even as we were still establishing the optimal doses for prenatal treatment. From E14 to parturition, an initial 2 groups of pregnant rats were either given daily injections of metyrapone prior to immobilization stress, or given injections of exogenous CORT (5 mg/kg) in lieu of immobilization stress, as described above. The offspring of these rats and their vehicle-treated/non-stressed controls were allowed to mature to late adolescence, at which point they were subject to CAPS stress, then tested in the fear conditioning and extinction paradigm Model 3, Task 2, Steps 5 and 11). However, in these groups we did not replicate the previous effect of PNS-CAPS-induced deficits in fear extinction. We believe that this was due to the use of DMSO as the prenatal vehicle, as described above, and we are currently repeating this experiment with oil and saline as respective vehicles.

We also examined changes in coping style as a result of prenatal stress and/or CAPS (Model 3, Task 1, Step 6; Task 2, Steps 5 and 6) substituting shock probe defensive burying for cognitive deficits (Model 3, Task 2, Step 6). Timed-pregnant female rats were singly housed throughout pregnancy. Half the pregnant females were immobilized daily for 1 hr, from day 14 of pregnancy until birth. Unstressed control pregnant females were left undisturbed during this same time period. On postnatal day (PD) 5, litters were culled to 8 pups each, maximizing the number of males retained, and weaned on PD 21. Upon weaning, male pups were pair-housed with a littermate until PD 51-53, at which time they were singly housed. A subset of the rats that received prenatal stress and a subset of those that did not were then subjected to adult CAPS stress or served as unstressed controls. The combination of PNS and adult CAPS treatment resulted in 4 treatment groups: No PNS/No CAPS (i.e., unstressed controls; n=20), PNS/No CAPS (n=7), No PNS/CAPS (n=10), and PNS/CAPS (n=13). One day after CAPS (or the comparable control time, at PD 66-68), the rats were tested in the shock probe defensive burying test. They were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe.

The results are shown in **Figure 2**. CAPS-treated rats displayed a significant reduction in the amount of time spent burying ( $F(1,44)=4.323, p<0.05$ ). Likewise, these rats displayed an increase in immobility ( $F(1,44)=20.65, p<0.0001$ ). CAPS-treated rats displayed a significant decrease in the burying ratio, calculated as (time spent burying)/(time spent burying + time spent immobile)\* 100 ( $F(1,44)=11.02, p<0.01$ ), reflecting a change in coping strategy, from a predominantly active strategy, as seen in the non-CAPS groups, to a predominantly passive strategy. This further validates the utility of the CAPS treatment as a model of traumatic stress, and indicates that an important component of CAPS-induced behavioral pathology may be reflected in a shift from an adaptive active coping strategy to maladaptive passive coping. Further, in replication of the results in our initial publication, there was again no effect of PNS

alone on burying behavior or on immobility, nor was there an interaction between PNS and CAPS.

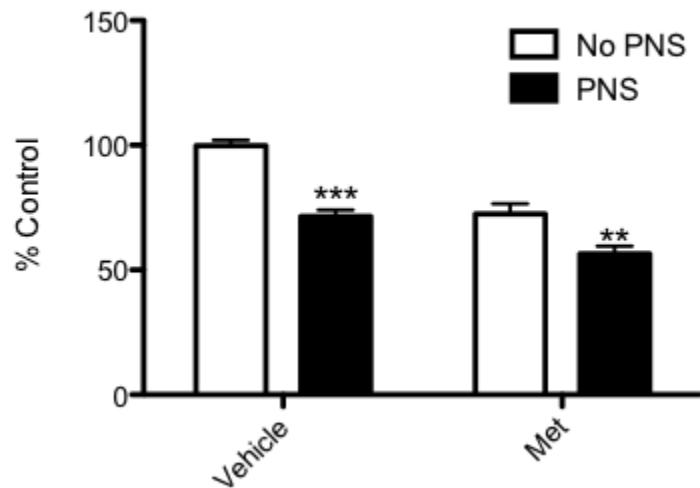


**Figure 2. Effects of prenatal stress and adult stress on passive and active coping in the shock probe defensive burying test. Data are the mean  $\pm$  SEM for 7 to 20 subjects.**

\* $P<0.05$ , significantly different from No CAPS.

\*\* $P<0.001$ , significantly different from No CAPS.

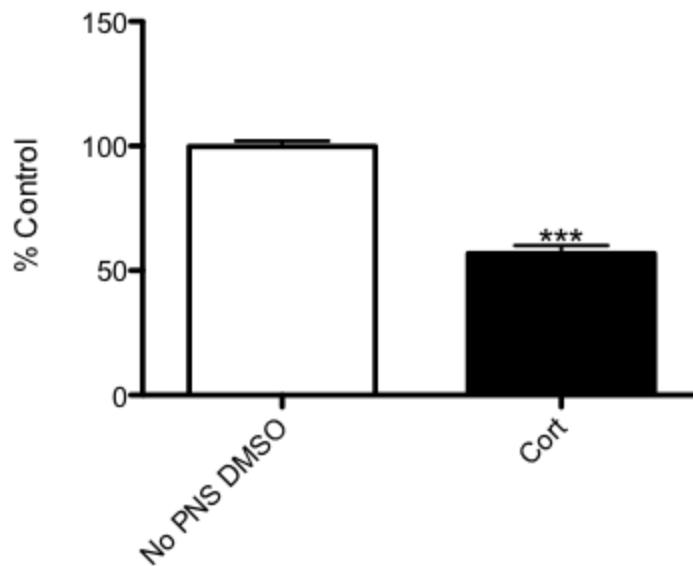
**Molecular biological analysis:** Concurrent with the behavioral experiments described above, we are investigating the role of prenatal CORT exposure on adult expression of TH mRNA (qPCR) and GR (ELISA). (Model 3, Tasks 1 and 2, Steps 14 and 15). Littermates of the rats used for behavioral experiments were allowed to grow to adulthood. We have shown that the effects of PNS on TH and GR expression are independent of adult stress, so these animals were not subject to CAPS stress (Task 2, Step 5). As shown in **Figure 3**, prenatal stress (PNS) was associated with decreased glucocorticoid receptor protein in the medial pre-frontal cortex. This replicated the finding that PNS decreases GR protein in the mPFC in our recently published studies. Interestingly, Metyrapone also had an effect, decreasing mPFC GR levels by itself. Similar to the behavioral results, we believe these molecular studies may also have been confounded by the use of DMSO as a vehicle. Therefore, a second study is currently underway.



**Figure 3. Effects of prenatal stress and metyrapone on glucocorticoid receptor protein in the medial prefrontal cortex.** Data are expressed as the mean  $\pm$  SEM.

\*\*,  $P<0.01$ , significantly different from No PNS.

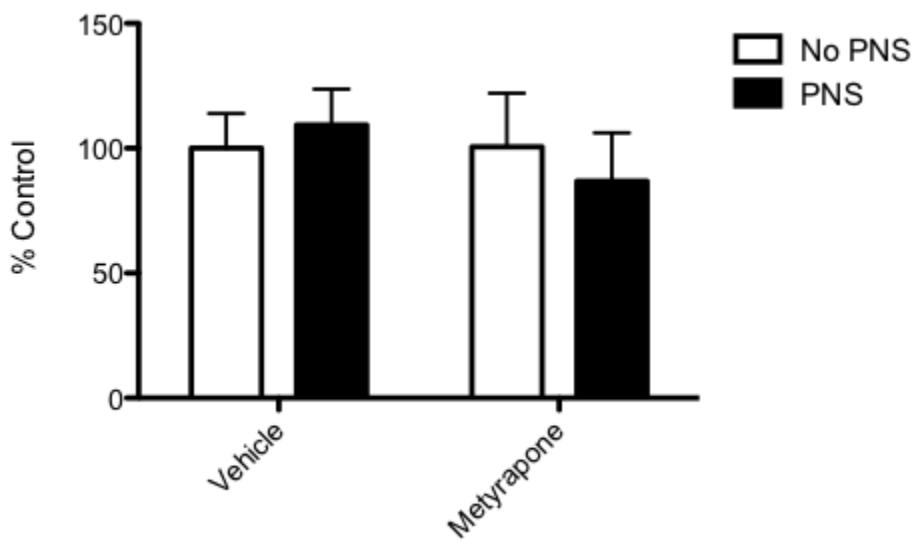
\*\*\*,  $P<0.001$ , significantly different from No PNS.



**Figure 4. Effects of prenatal corticosterone on glucocorticoid receptor protein in the adult medial prefrontal cortex.** Data are expressed as the mean  $\pm$  SEM.

\*\*\*,  $P<0.001$ , significantly different from vehicle control .

**Figure 4** shows the effects of prenatal treatment with corticosterone on glucocorticoid receptors in the mPFC of adult rats. Like PNS, corticosterone administered during the last week of pregnancy caused a significant decrease in glucocorticoid receptor expression in the adult offspring. These data support the idea that the effects of PNS on glucocorticoid receptors in adulthood are mediated by corticosterone.

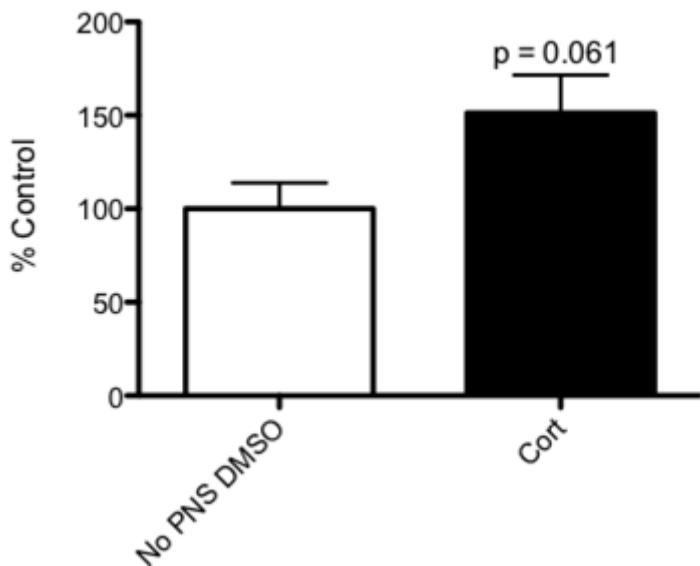


**Figure 5. Effects of prenatal stress and metyrapone on TH mRNA in the brainstem region containing the locus ceruleus.** Data are expressed as the mean  $\pm$  SEM.

\*\*,  $P<0.01$ , significantly different from No PNS.

\*\*\*,  $P<0.001$ , significantly different from No PNS.

**Figure 5** shows the effects of prenatal stress and metyrapone on TH mRNA in the brainstem region containing the locus ceruleus. In contrast to our recently published results, exposure to the DMSO vehicle and prenatal stress did not result in a reduction in TH mRNA. Moreover, metyrapone did not affect TH mRNA. As mentioned previously, we believe that this study may also have been confounded by the use of DMSO as a vehicle. Therefore, a second study is currently underway.



**Figure 6. Effects of prenatal corticosterone treatment on TH mRNA in the brainstem region containing the locus ceruleus.** Data are expressed as the mean  $\pm$  SEM.

We also observed that prenatal corticosterone treatment caused a marginally significant increase in TH mRNA (**Figure 6**). This is in contrast to the effects we observed in our recently published study on the effects of prenatal stress, which showed that maternal stress was associated with significant decreases in TH mRNA in the adult brainstem. Thus, corticosterone may not mediate this effect of prenatal stress. As mentioned previously, we are repeating these studies.

#### KEY RESEARCH ACCOMPLISHMENTS:

- We have established a dose of corticosterone that mimics the levels of corticosterone in mothers and pups exposed to prenatal stress.
- We have established a dose of metyrapone that blocks the increase in corticosterone induced by prenatal stress.
- We found that adult traumatic stress, independent of prenatal stress, causes changes in active and passive coping in the shock probe defensive burying test.
- We found that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex.
- We also found that, unlike prenatal stress, prenatal corticosterone had no effect on TH mRNA, suggesting that prenatal stress produces its effects on TH mRNA through a mechanism independent of corticosterone.

## **REPORTABLE OUTCOMES:**

Green MK, Rani CS, Joshi A, Soto-Piña AE, Martinez PA, Frazer A, Strong R, Morilak DA. Prenatal stress induces long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress. *Neuroscience*. 29;192:438-451, 2011.

Green MK, Joshi A, Frazer A, Strong R, Morilak DA (2010) Prenatal stress increases stress-reactivity and impairs fear extinction after adult stress: A model of PTSD. *Soc Neurosci Abstr* 36 Online Program no. 809.11

Green MK, Joshi A, Shah A, Frazer A, Strong R, Morilak DA (2011) Effects of prenatal stress and combined chronic plus acute adult stress on anxiety-like behavior and evoked HPA axis activity in rats. *Soc Neurosci Abstr* 37, Online Program no. 190.06.

Bingham B, Kaddapakam S, Strong R, Morilak DA (2011) Potential role of corticosterone in the long-lasting effects of prenatal stress. *Soc Neurosci Abstr* 37, Online Program no. 190.09.

**CONCLUSION:** During this year we began testing the second of four models of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We have established a dose of corticosterone that mimics the levels of corticosterone in mothers and pups exposed to prenatal stress. We have established a dose of metyrapone that blocks the increase in corticosterone induced by prenatal stress. We found that adult traumatic stress, independent of prenatal stress, causes changes in active and passive coping in the shock probe defensive burying test. We found that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex. We also found that, unlike prenatal stress, prenatal corticosterone had no effect on TH mRNA, suggesting that prenatal stress produces its effects on TH mRNA through a mechanism independent of corticosterone. Experiments are underway to replicate each of these experiments.

## **APPENDICES:**

**Reprint:** Green MK, Rani CS, Joshi A, Soto-Piña AE, Martinez PA, Frazer A, Strong R, Morilak DA. Prenatal stress induces long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress. *Neuroscience*. 29;192:438-451, 2011.

## **SUPPORTING DATA:**

Shown in the body of the report.

## PRENATAL STRESS INDUCES LONG TERM STRESS VULNERABILITY, COMPROMISING STRESS RESPONSE SYSTEMS IN THE BRAIN AND IMPAIRING EXTINCTION OF CONDITIONED FEAR AFTER ADULT STRESS

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**Abstract**—Stress is a risk factor for the development of affective disorders, including depression, post-traumatic stress disorder, and other anxiety disorders. However, not all individuals who experience either chronic stress or traumatic acute stress develop such disorders. Thus, other factors must confer a vulnerability to stress, and exposure to early-life stress may be one such factor. In this study we examined prenatal stress (PNS) as a potential vulnerability factor that may produce stable changes in central stress response systems and susceptibility to develop fear- and anxiety-like behaviors after adult stress exposure. Pregnant Sprague–Dawley rats were immobilized for 1 h daily during the last week of pregnancy. Controls were unstressed. The male offspring were then studied as adults. As adults, PNS or control rats were first tested for shock-probe defensive burying behavior, then half from each group were exposed to a combined chronic plus acute prolonged stress (CAPS) treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/d, 14 days) followed on day 15 by a single session of sequential acute stressors (social defeat, immobilization, cold swim). After CAPS or control treatment, different groups were tested for open field exploration, social interaction, or cued fear conditioning and extinction. Rats were sacrificed at least 5 days after behavioral testing for measurement of tyrosine hydroxylase (TH) and glucocorticoid receptor (GR) expression in specific brain regions, and plasma adrenocorticotropic hormone (ACTH) and corticosterone. Shock-probe burying, open field exploration and social interaction were unaffected by any treatment. However, PNS elevated basal corticosterone, decreased GR protein levels in hippocampus and prefrontal cortex, and decreased TH mRNA expression in noradrenergic neurons in the dorsal pons. Further, rats exposed to PNS plus CAPS showed attenuated extinction of cue-conditioned fear. These results suggest that PNS induces vulnerability to subsequent adult stress, resulting in an enhanced fear-like behavioral profile, and dysregulation of brain noradrenergic and hypothalamic–pituitary–adrenal axis (HPA) activity. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: ACTH, adrenocorticotropic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA axis, hypothalamic–pituitary–adrenal axis; HR, high responder rats; ITI, inter-trial interval; LC, locus coeruleus; NE, norepinephrine; PD, postnatal day; PNS, prenatal stress; PTSD, posttraumatic stress disorder; TH, tyrosine hydroxylase; WKY, Wistar-Kyoto rats.

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**Key words:** prenatal stress, traumatic stress, vulnerability, fear extinction, tyrosine hydroxylase, HPA axis.

Affective disorders, such as depression, post-traumatic stress disorder (PTSD) and other anxiety disorders, have long been considered to be stress-related/stress-initiated disorders. Responses to acute stressors are thought to be adaptive in the short term by increasing, for example, access to energy stores, increasing cardiovascular tone, and enhancing behavioral response capabilities. However, when these systems are repeatedly activated, as with chronic stress, dysregulation of a number of hormonal and neurotransmitter systems may occur (McEwen, 2003). Chronic stress is a risk factor, and possibly a causal factor, in the development of depression and anxiety disorders (Kendler et al., 1999; Koenen et al., 2002, 2007; Gilmer et al., 2005; Jordanova et al., 2007). Indeed, a number of physiological and anatomical alterations associated with chronic stress are hallmarks of depression and anxiety disorders (Board et al., 1956; Nemeroff et al., 1984, 1991; Gold et al., 1986; Holsboer et al., 1986; Weisse, 1992; Heuser et al., 1998; Arborelius et al., 1999; Manji et al., 2001; McEwen, 2003). In addition to cumulative or chronic stress, severe acute stress is also associated with mood and anxiety disorders, most prominently with PTSD. (Jordanova et al., 2007; American Psychiatric Association, 2000). However, not all individuals exposed to chronic or acute-traumatic stress in adulthood develop depression or anxiety disorders, suggesting that some other factor or factors, either genetic, epigenetic, or experience-based, contribute to susceptibility to develop affective disorders. Therefore, to fully understand the mechanisms underlying these disorders, it is insufficient to simply examine the response to stressors. Rather, the factors involved in predisposing for failure to recover from the normal response to stress must be identified (Yehuda and LeDoux, 2007).

Early life stress is one potential factor. For example, early life stress is a risk factor for PTSD, specifically, a history of trauma, childhood abuse/neglect, low education and IQ, low socio-economic status, or loss of a parent in childhood (Bremner et al., 1993; Breslau et al., 1999; Widom, 1999; Koenen et al., 2002, 2007). In rodents, prenatal stress (PNS) produces several behavioral and physiological changes that may be indicative of later stress vulnerability (e.g., Weinstock et al., 1992; Valle et al., 1997; Lemaire et al., 2000).

Two of the most prominent systems involved in stress adaptation, the brain noradrenergic system and the hypothalamic–pituitary–adrenal (HPA) axis, have also been implicated in stress-related pathology. Norepinephrine (NE) is released in response to stress (Morilak et al., 2005; Aston-Jones et al., 1999), chronic stress alters noradrenergic signaling (Buffalari and Grace, 2009; Kitayama et al., 2008; Ma and Morilak, 2005), and noradrenergic dysregulation is reported in numerous affective disorders, including depression and PTSD (Ressler and Nemeroff, 1999; Strawn and Geraciotti, 2008). Likewise, the HPA axis is activated in response to acute stress, resulting in release of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) and this response is altered after chronic stress (Dallman, 1993; Ma and Morilak, 2005). Furthermore, HPA axis dysregulation is a consistent component of several affective disorders, including depression, panic disorders, obsessive-compulsive disorder, and PTSD (Nemeroff et al., 1984; Gold et al., 1986; Souetre et al., 1988; Abelson et al., 2007; Kluge et al., 2007; Mason et al., 1986; Pitman and Orr, 1990).

It is being increasingly recognized that changes in executive function and cognitive capability are also prominent features of mood and anxiety disorders (Beck, 1976; Beck et al., 1987; Mathews and MacKintosh, 1998; Coles and Heimberg, 2002). Moreover, in the context of stress, both the brain noradrenergic system and the HPA axis are involved in regulation and dysregulation of cognitive processes such as learning and memory (de Quervain et al., 2009), including specifically conditioned fear and extinction learning (McIntyre et al., 2002; Mueller et al., 2008; Gourley et al., 2009). Impaired cognition, maladaptive fear responses, and impaired extinction of learned fear are primary symptoms of a number of affective disorders, with these fear-related symptoms being most relevant to anxiety disorders such as panic disorder, phobias, obsessive-compulsive disorder, and PTSD (Sutker et al., 1995; Fossetti et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangarathnam and Asbjørnsen, 2007; Blechert et al., 2007; Wessa and Flor, 2007). Therefore, it is possible that the mechanisms by which vulnerability factors such as prenatal stress may induce long-lasting susceptibility to develop psychopathology upon adult stress exposure could include dysregulation of the HPA axis and/or brain noradrenergic system, resulting specifically in maladaptive responses to fear-provoking stimuli and an impaired ability to extinguish fear responses in non-stressful conditions.

Thus, the purpose of the present study was to examine neurobiological correlates of adult stress vulnerability induced by PNS exposure. We measured the effects of PNS followed by a combined chronic plus acute prolonged stress (CAPS) treatment as adults, on tyrosine hydroxylase (TH) expression in the locus coeruleus (LC) and adrenal medulla, HPA status, and glucocorticoid receptor (GR) protein levels in the prefrontal cortex (PFC) and hippocampus. In the same rats, we also tested the vulnerability of PNS-exposed adult rats to develop fear or anxiety-like behaviors following exposure to CAPS treatment, on measures of acute stress reactivity, social interaction,

fear conditioning, and extinction. We hypothesized that PNS exposure would produce stable, long-term changes in central and peripheral stress response systems, and a vulnerability to subsequent adult stress exposure such that the behavioral impact of adult stress would be greater. Portions of this work have been presented in abstract form (Green et al., 2010).

## EXPERIMENTAL PROCEDURES

### Animals

Timed-pregnant (6 days pregnant upon arrival) female Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were singly housed throughout pregnancy. On postnatal day (PD) 5, litters were culled to eight pups each, maximizing the number of males retained (typically, three to six per litter), and weaned on PD 21. Upon weaning, male pups were pair-housed with a littermate until PD 41–45, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages ( $25 \times 45 \times 15 \text{ cm}^3$ ) on a 12/12 h light-dark cycle (lights on at 7:00 h) with food and water available *ad libitum*. In total, 141 adult male offspring (from 63 litters—33 stressed and 30 unstressed) were used in these experiments. In addition, for the social defeat procedure, six adult male Long–Evans rats (Harlan), weighing at least 400 g, were used as defeaters. They were housed, together with an ovariectomized female, in large resident cages ( $80 \times 55 \times 40 \text{ cm}^3$ ) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

### Prenatal stress treatment

After 1 week in the housing facility, half of the pregnant females were immobilized daily for 1 h, from day 14 of pregnancy until birth (8–9 days). Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement. Unstressed control pregnant females were left undisturbed during this same time period.

### Shock-probe defensive burying test

At PD 41–43, a subset of the offspring ( $n=60$ ) from both groups were tested in the shock probe defensive burying test. This was to evaluate potential differences in active and passive behavioral stress-reactivity as a consequence of the prenatal stress treatment prior to any exposure to adult stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. After the shock-probe defensive burying test, these animals were individually housed. Likewise, rats not tested in the shock-probe defensive burying test were also individually housed at this same time point.

## Adult stress treatment: chronic plus acute prolonged stress

Beginning between PD 46 and 54, half of the rats that received prenatal stress ( $n=35$ ) and half that did not ( $n=34$ ) received CAPS treatment, which consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min cold swim. For social defeat, the ovariectomized Long–Evans female was removed from the resident cage, and the test rat was placed into the resident cage. Typically within 10–30 s, the resident Long–Evans male rat will attack and defeat the smaller “intruder” Sprague–Dawley test rat. Once defeat occurred, with “defeat” defined as the test rat assuming a supine posture and the resident expressing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting the test rat from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization was then conducted, as described above, for 30 min. Finally, cold swim was accomplished by placing the rat in a cylindrical tank (30 cm diam×60 cm) filled to a depth of 30 cm with water at 18 °C. The combination of PNS and adult CAPS treatment resulted in four treatment groups: No PNS/No CAPS (i.e. unstressed controls), PNS/No CAPS, No PNS/CAPS, and PNS/CAPS.

## Neurochemical and hormonal analyses

All of the rats were sacrificed by rapid decapitation 5–10 days after the last stress day, or at the equivalent time for controls. Trunk blood was collected into tubes containing 10 mM EDTA and was centrifuged at 4000×g for 15 min at 4 °C for the separation and collection of plasma. Plasma was stored at –80 °C until use. Brains were removed, placed in a brain matrix on ice, and the structures of interest dissected. For PFC, a 2 mm coronal section was cut, extending from the frontal pole to approximately plate 8 in the atlas of Paxinos and Watson (1986). The hippocampus was then separated from the lateral margins of the remaining cortex. For the pontine region containing the LC, the cerebellum was removed and the obex located. A slab was cut 3–5 mm anterior to the obex (plates 53–60), and the dorsal half was collected. Brain samples were rapidly frozen in 2-methylbutane on dry ice, and stored at –80 °C until assay.

**TH mRNA.** TH mRNA was measured in the dorsal pons containing the locus coeruleus and in the adrenal medulla by qPCR. Total RNA was isolated from tissues using the RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA) as described by the manufacturer. The RNA concentration was measured by spectroscopy at 260 nm using the Nanodrop ND-1000 instrument (NanoDrop Technologies, Inc., Wilmington, DE, USA). To check the integrity of RNA, RNA was denatured with 50% formamide loading buffer and run on an E-Gel EX 1% agarose gel using the iBase Power System (Invitrogen Corp., Carlsbad, CA, USA) and the 18S and 28S bands visualized. In all, 250–500 ng RNA was converted to cDNA using random hexamers and TaqMan Multi-scribe reverse transcriptase enzyme included in the High Capacity RNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA). Reactions included controls without the reverse transcriptase enzyme but with only RNA template, and negative control with the enzyme, but no template in 20  $\mu$ l volume. After 2-h incubation at 37 °C, the RNA in the reactions was considered completely converted to cDNA. In order to check the quality of cDNA, cDNA (1  $\mu$ l=5 ng) from all reactions was used as template for PCR with rat GAPDH primers and the Go-Taq Green PCR master mix (Promega Corp., Madison, WI, USA). No bands

were detected either in negative control (no template) or in the samples without the reverse transcriptase enzyme, but a 200 bp band was seen in all cDNA-containing samples upon agarose gel electrophoresis.

TH gene expression was then quantified by qPCR using the TaqMan gene expression master mix and TaqMan Gene Expression assay ID Rn00562500\_m1, consisting of a set of intron-spanning primers and FAM-labeled probe set for rat TH (Applied Biosystems Inc.) along with the cDNA, equivalent to 0.125–0.25 ng RNA. Assays were performed in triplicate and first validated using the Applied Biosystems Inc. PRISM 7900 Sequence Detection System in a 96-well format. Results were normalized to 18S rRNA, which was amplified simultaneously in the same samples using the primer-limited TaqMan VIC-MGB labeled 18S rRNA probe (Applied Biosystems Inc.). Real time PCR data were analyzed by the  $2^{-\Delta\Delta C_T}$  method. The average difference in quantification cycle threshold (Cq) of the target gene and the 18S control for each sample was calculated, and the relative expression of TH in other groups was calculated with respect to the value obtained for the no-stress control samples.

**TH protein.** TH protein was measured in the adrenal medulla using Western blot. Samples were thawed on ice and homogenized in RIPA buffer (radioimmunoprecipitation assay buffer: 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS with Sigma protease inhibitor cocktail (P8340, 1:100 dilution) and phenylmethylsulfonylfluoride (PMSF, 1 mM) added just prior to use). Homogenates were centrifuged at 16,000×g for 15 min at 4 °C and the supernatants transferred to fresh tubes. Protein concentration in the lysates was assessed by MicroBCA method (Pierce Inc., ThermoFisher Scientific, Rockford, IL, USA). Equal amounts of sample protein in 1× NuPage LDS sample buffer (Invitrogen) under denaturing condition were loaded on 4–12% NuPage Bis–Tris SDS gels (Invitrogen) and electrophoresed at 175 V for ~1 h. Proteins separated on the gel were transferred to polyvinylidene fluoride membranes using the iBlot transblot apparatus (Invitrogen). Membranes were probed by simultaneous addition of specific monoclonal antibodies to TH (Sigma, St. Louis, MO, USA) and  $\beta$ -actin (AbCam, Cambridge, MA, USA), followed by secondary antibody consisting of IRDye 800CW conjugated goat polyclonal anti-mouse IgG (LI-COR Biosciences, Lincoln, NE, USA), and the fluorescent signal was scanned and quantified using the Odyssey infrared imaging system (LI-COR). The ratio of TH to actin of each sample was calculated, and the relative expression of TH was computed as percentage of the no-stress control group.

**GR protein levels in the hippocampus and PFC.** GR protein levels were assayed in the hippocampus and the PFC using an ELISA-based TransAM kit (Active Motif, Carlsbad, CA, USA). Assays were performed according to manufacturer's instructions. Briefly, the brain tissue was homogenized in complete lysis buffer AM2, containing 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail using motorized pestles in 1.5 ml microtubes (RPI, Mount Prospect, IL, USA). All steps were conducted at 4 °C. After 30 min incubation on ice, the homogenates were centrifuged at 10,000×g for 10 min and the supernatants were transferred to fresh chilled tubes. Aliquots were frozen or used for protein assay using the BioRad Bradford protein assay. For the ELISA, 20  $\mu$ g protein was used in a 96-well format. The homogenates, in complete binding buffer containing 1 mM DTT and herring sperm DNA, were incubated in wells coated with immobilized oligonucleotide containing a consensus GR binding site (5'-GGTACAnnnTGTTCT-3'). The bound GR was then detected using a specific GR primary antibody and an HRP-conjugated secondary antibody followed by a colorimetric step for quantification by spectrophotometry in a plate reader. The absorbance at 450 nm after suitable blank correction was used to determine GR levels. The A450 values for each

stress group were calculated as percentage of the no-stress control group.

**Plasma ACTH and CORT levels.** Levels of circulating ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100  $\mu$ l plasma aliquots according to the manufacturer instructions (ImmunoChem double antibody hACTH assay, MP Biomedicals, Orangeburg, NY, USA). The detection limit of the assay is 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to manufacturer instructions (ImmunoChem Double antibody, corticosterone assay, MP Biomedicals). The assay detection limit was 8 ng/ml, and inter-assay variability was 8%.

### Behavioral measures

**Fear conditioning and extinction.** One day after the termination of CAPS treatment (or the comparable control period), a subset of rats in each group ( $n=98$ ; 23–25/group) was habituated to the two fear conditioning and extinction contexts for 15 min each. Context A is a 30.5  $\times$  25.4  $\times$  30.5 cm<sup>3</sup> square conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA; model # H10-11R-TC) with metal walls and a grid shock floor attached to a shock generator (Coulbourn, # H13-15). Context B is a modified chamber with black and white vinyl walls forming a circular enclosure and a smooth green vinyl floor placed over the shock grid. Both contexts are enclosed in a 58.4  $\times$  61  $\times$  50.8 cm<sup>3</sup> sound-attenuating chamber (Coulbourn, # H10-24T). Twenty-four hours after habituation, the rats received cued fear conditioning in Context A. Each rat was placed into the chamber and, after a 5 min acclimation period, experienced four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminating with a shock (0.7 mA, 0.5 s). The average inter-trial interval (ITI) was 120 s.

Seventy-two hours after fear conditioning, the rats experienced fear extinction training consisting of exposure to 10 trials of the tone alone (average ITI 120 s, for a total 27 min extinction session). Then, 24 h later, the rats were tested for retention of fear extinction during exposure to 10 trials of the tone alone. To avoid any contextual effects, all extinction and retention testing was conducted in Context B.

Freezing behavior was recorded and analyzed using the FreezeFrame and FreezeView software (ActiMetrics Software; Coulbourn Instruments # ACT-100). Freezing was defined as all behavior which fell below the motion index threshold of 10 and lasted at least 1 s. Freezing was measured during each 20-s tone presentation on the conditioning, extinction training and extinction retention days. The time spent freezing was then expressed as a percentage of each 20-s sampling period.

**Open field exploration.** One day after the termination of CAPS treatment (or comparable control period), the remaining rats ( $n=43$ ; 10–12/group) were tested for anxiety-like and exploratory behavior in an open field (60  $\times$  60  $\times$  40 cm<sup>3</sup>) under normal ambient laboratory lighting. The floor of the test arena was marked in a grid pattern of 36 squares, 10  $\times$  10 cm<sup>2</sup> each. The test rat was placed into the center of the open field and behavior was recorded for 5 min. The number of line crossings and time spent in the center zone (i.e. the inner 16 squares) were measured.

**Social interaction.** Twenty-four hours after open field testing, the rats were tested in the same arena for social interaction with a novel male con-specific, weight-matched to within  $\pm 5$  g of the test rat. The con-specific "stimulus rats" had all been previously habituated to interacting in the arena with other stimulus rats so that their behavior would be constant during testing. The amount of time that the test rat spent engaged in social behaviors (sniffing, chasing, climbing, etc.) was measured during the 5-min test.

### Statistical analyses

For the shock-probe defensive burying data, differences in immobility time and in active burying time between rats in the two prenatal stress conditions (prenatally stressed and controls) were analyzed by *t*-tests. All neurochemical and plasma hormone measures were analyzed by two-way ANOVA (prenatal stress  $\times$  adult stress). Similarly, for the open field and social interaction tests, group differences in the number of line crossings, time spent in the center zone of the open field, and social interaction time were each analyzed by a two-way ANOVA (prenatal stress  $\times$  adult stress). For the fear-conditioning and extinction tests, group differences in percent freezing were analyzed for each session by a three-way ANOVA (prenatal stress  $\times$  adult stress  $\times$  tone) with repeated measures over tone. To determine if there were differences in the retention of conditioned fear, freezing levels in response to the first tone presentation on the extinction training day were analyzed by two-way ANOVA (prenatal stress  $\times$  adult stress). Likewise, tone 1 was analyzed in the same way on the extinction retention day as a measure of retention of extinction that occurred the day before (see Milad et al., 2004; Vidal-Gonzalez et al., 2006; Muigg et al., 2008). Following the primary ANOVA, in order to better assess and compare the rate of extinction across groups, a non-linear regression analysis was performed. An exponential decay function was best-fit to the freezing data for each rat, from which the rate constants (*k*) were derived. The rate constants for each treatment group were then compared by two-way ANOVA (prenatal stress  $\times$  adult stress). On the extinction training day, an increase in freezing was always observed from tone 1 to tone 2. Thus, in order to capture the true rate of extinction from the peak level of freezing, and also to obviate any potential group differences in extinction attributable solely to different starting points on tone 1, tone 1 was not included in the regression analysis. Further, cases for which a line could not be fit to the data were excluded from the regression analysis, resulting in the exclusion of one to two animals per group. Upon examination of these cases, no consistent pattern could be discerned, and the data from these cases were included in the primary ANOVA for freezing data. In all analyses, significance was determined at  $P<0.05$ . Sources of significant main effects or interactions were then determined by analysis with the Newman-Keuls post hoc test.

## RESULTS

### Shock-probe defensive burying test

Prior to administering the adult stress treatment, there were no significant differences between rats that received prenatal stress and rats that did not in either time spent immobile (Table 1;  $t_{58}=0.381$ ,  $P>0.05$ ), nor in time spent burying the shock probe ( $t_{58}=0.599$ ,  $P>0.05$ ).

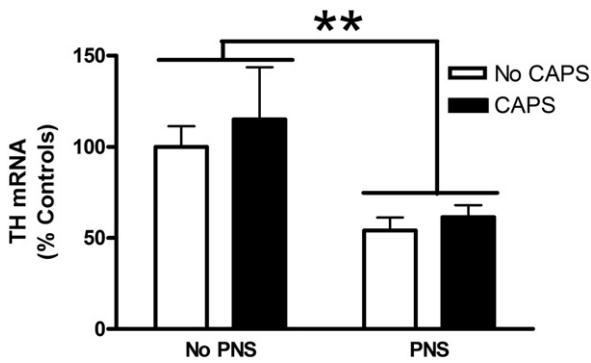
### TH expression in the locus coeruleus and adrenal medulla

Prenatal stress significantly reduced TH mRNA expression in homogenates of rostral pons containing the LC, irrespective of exposure to CAPS (Fig. 1;  $F_{(1,66)}=9.745$ ,

**Table 1.** Shock probe defensive burying behavior before adult stress

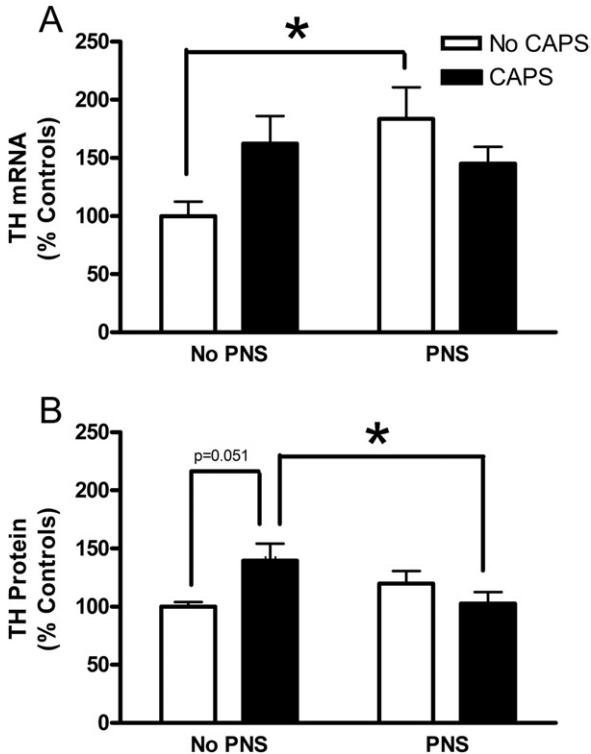
	Controls	PNS
Immobilization time (s)	$150.9 \pm 31.95$	$166.2 \pm 24.99$
Burying time (s)	$97.77 \pm 20.11$	$81.56 \pm 18.24$

Data expressed as mean  $\pm$  SEM,  $n=28$ –32/group.

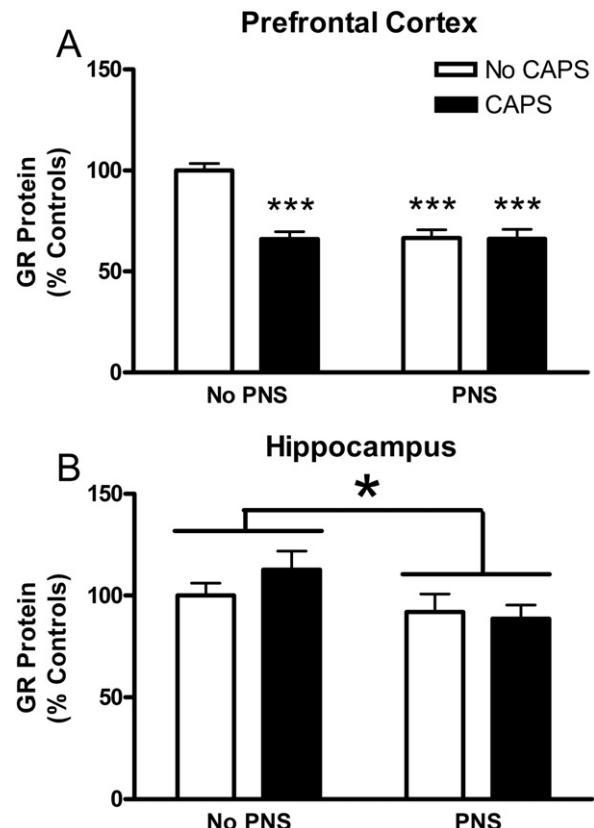


**Fig. 1.** Tyrosine hydroxylase mRNA expression in the rostral pons containing the locus coeruleus. Prenatal stress produced a significant decrease in TH mRNA expression in the LC region. \*\* main effect of PNS,  $P=0.003$ . Data expressed as mean percent of controls $\pm$ SEM,  $n=17$ –19/group.

$P<0.01$ ). There was no main effect of CAPS ( $F_{(1,66)}=0.488$ ,  $P>0.05$ ) nor an interaction between PNS and CAPS ( $F_{(1,66)}=0.061$ ,  $P>0.05$ ). However, effects in the adrenal medulla were different (Fig. 2). For TH mRNA, there was no main effect of PNS (Fig. 2A;  $F_{(1,33)}=2.606$ ,  $P>0.05$ ) nor was there an effect of CAPS ( $F_{(1,33)}=0.343$ ,  $P>0.05$ ), but there was a significant PNS $\times$ CAPS interaction ( $F_{(1,33)}=6.06$ ,  $P<0.05$ ) such that PNS alone induced



**Fig. 2.** Tyrosine hydroxylase mRNA and protein expression in the adrenal medulla. (A) PNS alone significantly increased TH mRNA expression in the adrenal medulla compared to unstressed controls, \*  $P=0.03$  by Newman–Keuls post hoc comparisons,  $n=8$ –10/group. (B) CAPS alone significantly increased TH protein levels compared to both PNS/CAPS (\*  $P=0.04$ ) and unstressed controls ( $P=0.051$ ). Data expressed as mean percent of controls $\pm$ SEM,  $n=13$ /group.



**Fig. 3.** Effects of PNS and CAPS on glucocorticoid receptor (GR) expression. (A) There was significantly reduced GR protein expression in the prefrontal cortex after either PNS or CAPS alone, as well as the combined PNS/CAPS treatment. \*\*\*  $P=0.0001$  compared to unstressed controls,  $n=18$ –20/group. (B) PNS modestly but significantly reduced GR protein expression in the hippocampus. \* Main effect of PNS,  $P=0.04$ . Data expressed as mean percent of unstressed controls $\pm$ SEM,  $n=14$ –16/group.

significantly higher TH mRNA expression compared to controls receiving no PNS. There was also a non-significant elevation of TH mRNA in the group that received CAPS alone ( $P=0.09$ ). Likewise, for TH protein, there was no significant main effect of PNS (Fig. 2B;  $F_{(1,48)}=0.664$ ,  $P>0.05$ ) nor of CAPS ( $F_{(1,48)}=1.107$ ,  $P>0.05$ ), but a significant PNS $\times$ CAPS interaction ( $F_{(1,48)}=7.272$ ,  $P<0.01$ ). Post hoc analyses reveal that rats exposed to CAPS alone had more adrenal TH protein than did control rats ( $P$  approaching significance at 0.051) or rats exposed to PNS plus CAPS.

#### GR levels in the mPFC and hippocampus

In the prefrontal cortex, both PNS and CAPS significantly decreased GR protein (Fig. 3A;  $F_{(1,70)}=16.906$ ,  $P<0.0001$  for PNS;  $F_{(1,70)}=18.177$ ,  $P<0.0001$  for CAPS) with no apparent additive effect of the two treatments, resulting also in a significant interaction of PNS and CAPS ( $F_{(1,70)}=17.171$ ,  $P<0.0001$ ). Post hoc analyses revealed that the three stress conditions (PNS, CAPS, PNS/CAPS) did not differ from each other. By contrast, in the hippocampus, only a main effect of prenatal stress was evi-

dent (Fig. 3B;  $F_{(1,55)}=4.368, P<0.05$ ), causing a significant decrease in GR protein, but no main effect of CAPS ( $F_{(1,55)}=0.37, P>0.05$ ) nor an interaction of PNS and CAPS ( $F_{(1,55)}=1.06, P>0.05$ ).

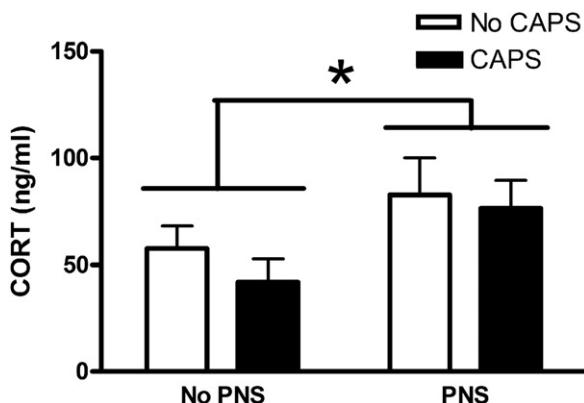
### Plasma ACTH and CORT

There were no effects of PNS or CAPS on basal plasma ACTH ( $F_{(1,100)}=0.091$  for PNS,  $F_{(1,100)}=0.003$  for CAPS,  $P>0.05$ ), nor was there a significant interaction ( $F_{(1,100)}=1.692, P>0.05$ ). However, prenatal stress induced a significant, long-term elevation in basal CORT (Fig. 4;  $F_{(1,99)}=5.068, P<0.05$ ). Adult stress had no effect on basal CORT ( $F_{(1,99)}=0.702, P>0.05$ ) nor was there a significant interaction between PNS and CAPS ( $F_{(1,99)}=0.123, P>0.05$ ), probably because sacrifice and trunk blood collection for plasma measures took place 5–10 days after the termination of the adult stress treatment.

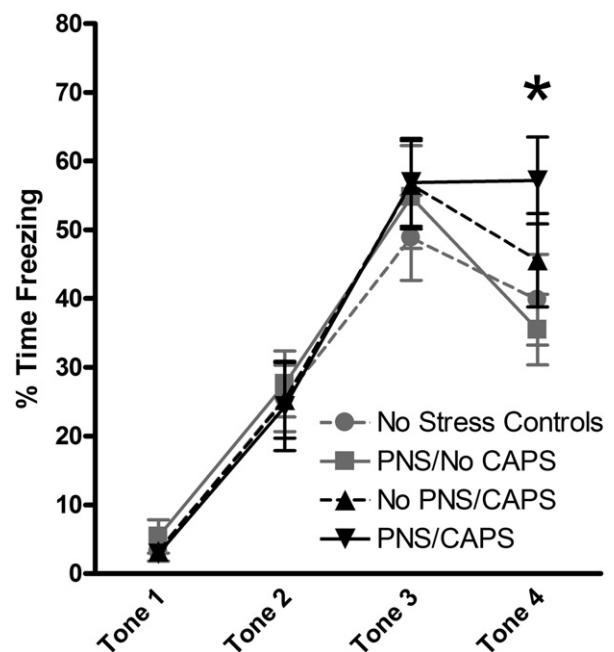
### Fear conditioning and extinction

On all 3 days, there was the expected main effect of Tone ( $F_{(3,276)}=107.712, P<0.001$  for conditioning;  $F_{(9,828)}=38.875, P<0.001$ ;  $F_{(9,828)}=21.271, P<0.001$  for extinction training and retention, respectively), confirming that the conditioning and extinction protocols were effective. Rats exposed specifically to PNS plus CAPS displayed enhanced fear conditioning and impaired extinction. For fear conditioning, there was a significant interaction between CAPS and Tone (Fig. 5;  $F_{(3,276)}=2.813, P<0.05$ ). Post hoc analyses revealed that the PNS/CAPS group expressed significantly higher levels of freezing than did the PNS group on tone 4. There were no other main effects or interactions during conditioning.

On the test day, freezing in response to tone 1 alone was first analyzed to determine if there were any differences in the retention of conditioning from the previous day, and there were not. There were no main effects of PNS ( $F_{(1,92)}=1.317, P>0.05$ ), or CAPS ( $F_{(1,92)}=0.4607, P>0.05$ ), nor an interaction ( $F_{(1,92)}=0.1656, P>0.05$ ). Next, the analysis of the subsequent course of extinction

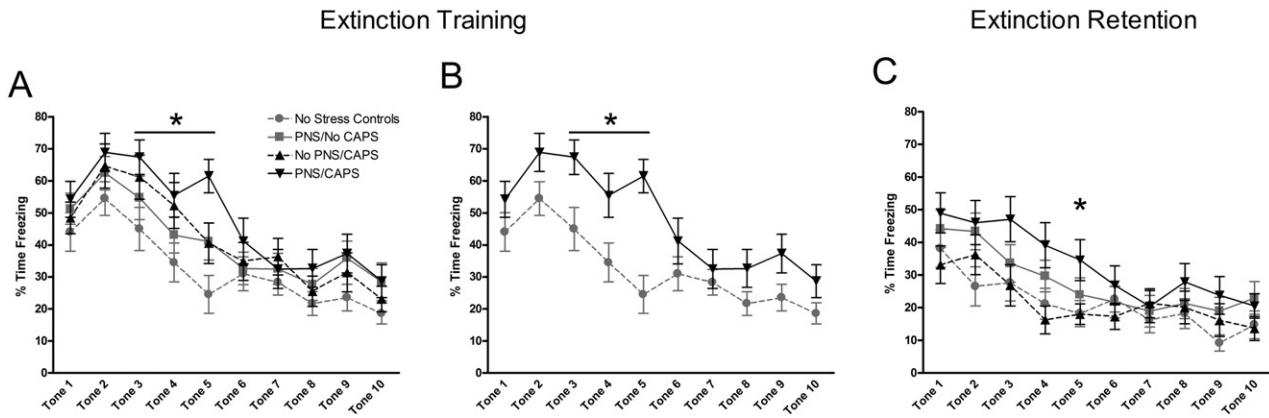


**Fig. 4.** Effects of PNS and CAPS on basal corticosterone. PNS-treated rats had significantly higher basal levels of circulating corticosterone. \* Main effect of PNS,  $P=0.03$ . Data expressed as mean $\pm$ SEM,  $n=25$ –27/group.



**Fig. 5.** Combined PNS/CAPS treatment enhanced the expression of learned fear. Rats that received PNS/CAPS displayed enhanced freezing on tone 4 of fear conditioning. \* PNS/CAPS vs. PNS/No CAPS,  $P=0.009$ . Data expressed as mean $\pm$ SEM,  $n=23$ –25/group.

training showed that there was a significant effect of CAPS (Fig. 6A;  $F_{(1,92)}=3.97, P<0.05$ ) and a significant interaction between CAPS and Tone ( $F_{(9,828)}=1.9, P<0.05$ ). Additionally, the main effect of PNS approached significance ( $F_{(1,92)}=3.18, P=0.078$ ). Post hoc analyses revealed that the effect of CAPS was manifest as a delay in extinction (i.e. more tones required for extinction), as CAPS-treated rats had significantly higher freezing on tones 3–5 compared to unstressed controls (Fig. 6A). Subsequent post hoc comparisons between groups indicated that this was driven largely by a delayed extinction profile specifically in the combined PNS/CAPS group, in which freezing behavior remained elevated longer than in the other groups. The PNS/CAPS group had significantly more freezing than unstressed controls on tones 3–5, and more than both the PNS-only and CAPS-only groups on tone 5 (Fig. 6A, B). To further assess differences specifically in the rate of extinction, an exponential decay function was fit to each rat's freezing data, and the resulting rate constants ( $k$ ) were compared by two-way ANOVA. Confirming the results of the primary ANOVA, there was a significant effect of CAPS ( $F_{(1,89)}=7.522, P<0.01$ ), reflecting a slower rate of extinction, that was especially evident in the PNS/CAPS group. Post hoc analyses showed that the PNS/CAPS group had significantly slower rates of extinction than both the unstressed control group and the PNS only group (Fig. 7A, B). These analyses suggest that the extinction deficit induced by CAPS, and most prominently in the PNS/CAPS group, was driven primarily by a decrease in the rate of extinction. By contrast, also as seen in Fig. 7, the effect of PNS was primarily to elevate freezing (i.e. increased fear) without affecting the rate of extinction.



**Fig. 6.** Combined PNS/CAPS treatment impaired extinction. (A) CAPS caused a delay in extinction, as freezing in the CAPS-treated rats remained significantly elevated during tones 3–5 compared to non-CAPS-treated rats (\*  $P<0.05$ , post hoc comparisons by Newman–Keuls). (B) Specific comparison of extinction in the PNS/CAPS and control groups showed that freezing in the PNS/CAPS group also remained significantly elevated during tones 3–5 compared to unstressed controls (\*  $P<0.05$ , post hoc comparisons by Newman–Keuls), and during tone 5 compared to both the No PNS/CAPS and PNS/No CAPS groups (for clarity, only the PNS-CAPS and control groups are shown). (C) During extinction retention testing on the following day, overall freezing was elevated by PNS alone (\* main effect of PNS,  $P<0.05$ ). Although the PNS/CAPS group again appeared to be the most affected, that specific comparison was not significant ( $P<0.10$ ). Data in all panels expressed as mean $\pm$ SEM,  $n=23$ –25/group.

PNS treatment also elevated freezing during extinction retention, tested on the following day (Fig. 6C). Freezing in response to tone 1 was first analyzed, as above. There was no significant effect of CAPS ( $F_{(1,92)}=0.0002$ ,  $P>0.05$ ) nor an interaction between PNS and CAPS ( $F_{(1,92)}=0.6936$ ,  $P>0.05$ ). However, the main effect of PNS approached significance ( $F_{(1,92)}=3.505$ ,  $P=0.064$ ), suggesting that rats exposed to PNS had modestly elevated freezing even at the outset of the extinction retention day. Subsequent analysis of the full course of extinction retention by ANOVA revealed only a significant main effect of PNS ( $F_{(1,92)}=5.798$ ,  $P<0.05$ ). The PNS/CAPS group once again appeared to be most affected (Fig. 6C), although this specific comparison was not significant ( $P<0.10$ ). That PNS induced an overall elevation in freezing without affecting the trajectory of extinction on retention day was confirmed by analyzing the rate constants derived from the exponential decay curves fit to the extinction retention data, for which there were no significant differences (Fig. 7C, D). Thus, unlike the effect of CAPS, and especially of PNS/CAPS, on the rate of extinction during training, the effect of PNS on extinction retention was an overall elevation in freezing, evident from trial 1 on.

#### Open field exploration and social interaction

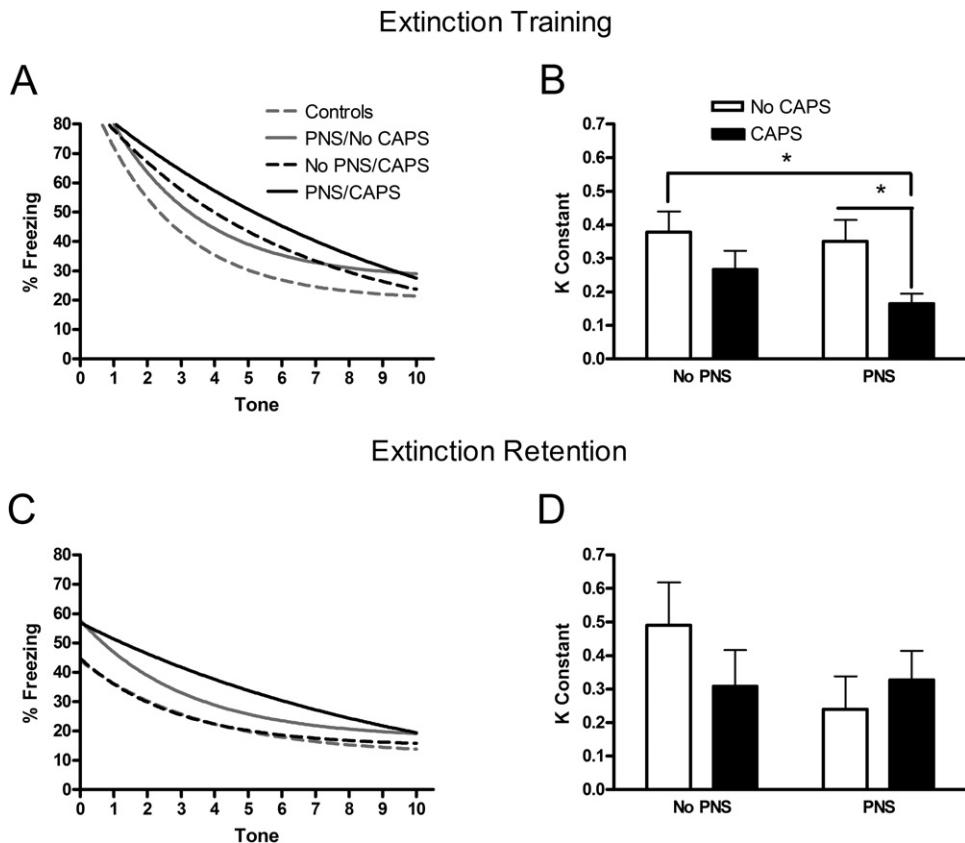
In the open field test, there were no significant main effects of PNS or CAPS on number of line crossings (Table 2;  $F_{(1,39)}=0.057$  for PNS,  $F_{(1,39)}=0.028$  for CAPS,  $P>0.05$ ) or time spent in the center zone ( $F_{(1,39)}=2.058$  for PNS,  $F_{(1,39)}=0.119$  for CAPS,  $P>0.05$ ). Likewise, there was no significant interaction between PNS and CAPS on either measure ( $F_{(1,39)}=0.195$ ,  $P>0.05$  for line crossings;  $F_{(1,39)}=3.303$ ,  $P>0.05$  for center time). Similarly, in the social interaction test, there were no significant main effects or interactions of PNS and CAPS on time spent interacting with a novel conspecific (Table 2;  $F_{(1,39)}=0.261$

for PNS,  $F_{(1,39)}=0.578$  for CAPS,  $F_{(1,39)}=0.041$  for PNS $\times$ CAPS, all  $P>0.05$ ).

## DISCUSSION

The hypothesis tested in this experiment was that prenatal stress produces a vulnerability to severe stressors in adulthood, such that those rats that experienced prenatal stress would exhibit a greater detrimental behavioral effect following adult stress, which might be accounted for by specific neurochemical changes in the brain and/or periphery. We found that PNS induced stable baseline alterations on several neurochemical parameters independent of adult stress exposure, and also induced a greater sensitivity to adult stress in some, but not all of the neurochemical and behavioral measures. We found that adult stress alone impaired extinction, and that effect was exacerbated by PNS. Thus, we conclude that prenatal stress exposure induces long term and stable changes in brain and peripheral stress response systems that represent a potential vulnerability to subsequent adult stress.

The rats were stressed during the last week of pregnancy (from E14 to birth), a critical period in development of the fetal HPA system, and also of potential sensitivity to maternal glucocorticoids. GR and mineralocorticoid receptor (MR) receptors are expressed in the developing rat brain at E13–E16 (Kitraki et al., 1996; Diaz et al., 1998), and fetal production of CORT, as well as maternal CORT concentrations, increase from E16 to E19 (Dupouy et al., 1975). Further, placental expression and activity of the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase 2, which protects the fetus from maternal CORT by metabolizing it to inactive 11-dehydrocorticosterone, decreases from E16 to birth (Waddell et al., 1998). Functionally, a comparison of stress exposure during the second and third weeks of gestation found a lasting change in HPA regulation only in



**Fig. 7.** Effects of PNS and CAPS on rates of extinction. Exponential decay curves were fit to each rat's freezing data, providing a rate of decay ( $k$  constant) for each animal. The  $k$ -values were then compared by two-way ANOVA to determine group differences in the rate of extinction. (A) For the purposes of illustration, exponential decay curves were fit to each group's mean data, representing the average rate of extinction for each group on training day. (B) In the analysis of  $k$ , CAPS produced a slower rate of extinction during training, with the PNS/CAPS rats displaying a significantly slower rate compared to the PNS alone and unstressed control groups (\*  $P < 0.05$  by Newman–Keuls post hoc comparison). (C) Exponential decay curves were fit to each group's mean data to illustrate the average rate of extinction on retention day. (D) There were no significant effects of PNS or CAPS on  $k$ , the rate constant for extinction on the retention day. Data expressed as mean  $\pm$  SEM,  $n=23$ –25/group.

the offspring of mothers stressed during the third week (Koenig et al., 2005).

In the present study, PNS induced a 50% reduction in TH mRNA in the dorsal pons, including noradrenergic neurons in the LC and subcoeruleus. This effect was specific to the brain, as there was, if anything, a modest elevation of TH mRNA and/or protein in the adrenal medulla after either PNS or CAPS. PNS also produced stable, long-term changes in the HPA axis. Adult offspring exposed to PNS had significantly elevated basal circulating CORT and reduced GR expression in the hippocampus and PFC, suggesting potential impairment of HPA negative feedback mechanisms. These neurochemical changes are consistent with effects of PNS reported previously in the

literature. For example, PNS has been shown to affect regulation of the HPA axis, including alterations in circadian rhythm (Koehl et al., 1999), elevated CORT, and delayed recovery after mild stress (Weinstock et al., 1992; Barbazanges et al., 1996; Valle et al., 1997; Koenig et al., 2005; Fan et al., 2009). PNS has also been shown to decrease neurogenesis (Lemaire et al., 2000), and the expression of both the GR and the MR in the hippocampus (Barbazanges et al., 1996; Koehl et al., 1999). As in the present study, these effects were all observed in adult offspring of dams that had been stressed during the final week of pregnancy, suggesting that the changes are life-long and could thus alter the response to subsequent chronic or traumatic stress in adulthood.

**Table 2.** Open field exploration and social interaction

	Controls	PNS alone	CAPS alone	PNS/CAPS
Open field line crossings	$84.5 \pm 14.76$	$74.3 \pm 15.88$	$80.4 \pm 12.28$	$83.45 \pm 16.27$
Open field center zone time (s)	$8.23 \pm 2.05$	$9.07 \pm 1.51$	$11.47 \pm 3.49$	$4.32 \pm 1.2$
Social interaction time (s)	$67.3 \pm 7.85$	$70.07 \pm 10.16$	$58.67 \pm 5.58$	$65.07 \pm 10.99$

Data expressed as mean  $\pm$  SEM,  $n=10$ –12/group.

Both PNS and CAPS also impacted behavioral responses to fear conditioning and extinction, although in different ways, and rats exposed to PNS plus CAPS had the greatest behavioral deficits. First, PNS/CAPS rats displayed slightly elevated freezing at the end of fear conditioning, due primarily to a failure to decrease freezing on the fourth trial. We have frequently observed that after a certain number of tone-shock pairings during fear conditioning, rats can start to shift to a more active coping response, exhibiting active escape behavior and less passive freezing, which was just beginning to occur on tone 4 in the other groups. Thus, the pattern of response seen on the last conditioning trial may indicate that the PNS/CAPS rats persisted in maintaining a more passive coping response to the mild acute stress.

Next, the detrimental effects of adult stress on extinction were manifest as a delay in the rate of extinction. PNS alone modestly elevated the overall level of freezing, whereas CAPS-treated rats had a significantly slower rate of extinction, and this was enhanced by prior PNS exposure. It is noteworthy that on the extinction training day, despite clear differences in the rate of extinction learning, all groups eventually achieved extinction by the end of the session. Having thus achieved extinction, there were no residual effects of CAPS treatment alone on the retention of extinction 1 day later. However, during testing for the retention of extinction, the PNS-treated rats, particularly the combined PNS/CAPS-treated rats, continued to display elevated levels of freezing, which was evident even on tone 1, and was maintained across trials, but with no differences in the extinction rate constants on the retention day. Thus, even though all groups reached equivalent levels of extinction on the training day, PNS-treated rats maintained an elevated level of fear on the following day. These results suggest that severe adult stress exposure alone can have a transient detrimental effect on the rate of extinction, which may be evident in the short-term consequences of traumatic stress exposure, but that these deficits can eventually be resolved with sufficient extinction training, until full extinction is achieved. However, a predisposing history of PNS exposure not only exacerbated the detrimental effect of adult stress on the process of extinction, but in itself it also induced a modest but persistent propensity to exhibit enhanced fear, seen as an increase in freezing during both extinction learning and retention, thus acting as a vulnerability factor.

A similar effect of PNS was reported previously (Markham et al., 2010). In that study, PNS-treated male rats displayed less freezing during conditioning, but more freezing during extinction training and retention compared to control male rats. The different effects observed during conditioning as compared to the present study may be due to differences in the PNS protocol. In the Markham study, PNS involved a variable stress procedure, whereas we used a repeated homotypic stressor (immobilization). Interestingly, in the Markham study, no effects of PNS were seen in females, in either conditioning or extinction. Thus, the factors involved in PNS-induced vulnerability

may be particularly relevant to human males facing combat exposure.

Impairments in the rate of extinction of conditioned fear are relevant to human neuropsychiatric disorders in which conditioned fear is a prominent component, such as PTSD, panic disorder, and phobias. In these disorders, there is an inability to extinguish a fear reaction and reinforcement of the fear response. For example, PTSD patients show impairments in extinction (Blechert et al., 2007; Wessa and Flor, 2007) similar to those found in our study, and they have difficulty suppressing fear responses in the presence of safety signals, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). Further, exposure therapy, a form of extinction training, is effective for approximately 50% of PTSD patients (Bradley et al., 2005), although it is considered one of the most successful treatments for PTSD. In the present study, initial impairments in extinction were seen in the CAPS-treated rats, but successful “recovery” was maintained once extinction was achieved. This may reflect the success of exposure therapy in a proportion of trauma-exposed humans. On the other hand, PNS exposure induced a long-term vulnerability to adult stress, reflected by enhanced impairment of extinction learning and retention, and elevated fear even after extinction. This may reflect the fact that, in humans, certain vulnerable individuals remain impaired and/or resistant to exposure therapy. Further, the differences in the effects of CAPS alone versus PNS plus CAPS may reflect the transition from an acute and transient, perhaps even adaptive response to stress, into a long-term PTSD-like state in vulnerable individuals.

The neurochemical systems in which changes were observed after PNS may provide clues to potential mechanisms underlying the subsequent vulnerability to adult CAPS exposure, and the resulting impairments specifically in the extinction of conditioned fear. TH is the rate-limiting step in catecholamine synthesis. Chronic down-regulation of TH in the forebrain-projecting noradrenergic neurons in the LC, including the sole source of NE input to both PFC and hippocampus, could reflect a reduced capacity for sustained NE release in these forebrain targets in the face of chronic or severe stress. Reduction in brainstem TH, and presumably in NE release, could be one mechanism underlying impaired extinction learning and retention after PNS/CAPS. NE neurotransmission has been implicated in both fear conditioning and extinction. During conditioning, NE release in the amygdala is correlated with the retention of fear memories (Galvez et al., 1996; Quirarte et al., 1998; McIntyre et al., 2002), which is impaired by  $\beta$ -adrenergic receptor blockade (Fu et al., 2008). Likewise, NE levels increase in the mPFC in response to emotionally salient stimuli (Feenstra et al., 2001; Mingote et al., 2004; Hugues et al., 2007), and extinction retention is also impaired by blockade of  $\beta$ -adrenergic receptors, and enhanced by administration of yohimbine, an  $\alpha_2$ -adrenergic autoreceptor antagonist that increases NE levels, prior to extinction training (Mueller et al., 2008; Cain et al., 2004).

Glucocorticoids also affect learning and memory, although in a more complex manner (see de Quervain et al.,

2009). Acute CORT administration immediately after training in a novel object recognition task enhanced consolidation and recognition (Roozendaal et al., 2006). By contrast, injections of GR agonist into the hippocampus immediately before retention testing in a Morris water maze impaired spatial memory (Roozendaal et al., 2004), and both of these effects were dependent on convergent noradrenergic signaling. Further, either chronic low-dose CORT or acute GR antagonists given after the first extinction exposure impaired extinction (Gourley et al., 2009). Thus, chronically elevated CORT, together with GR down-regulation and reduced NE release capacity in PFC may have all contributed to impaired extinction after PNS/CAPS.

Monoamines, including NE, have been implicated in both the stress response and in stress-related affective disorders, and antidepressants that affect the monoamines, serotonin, and NE, are the most effective pharmacological treatments for depression and anxiety disorders. However, while drugs targeting NE are effective therapeutically, evidence suggesting dysregulation of noradrenergic signaling in mood and anxiety disorders is less consistent, including alterations in NE levels and adrenergic receptor expression in depression, and reduced NE metabolite levels during the depressive phase of bipolar disorder (see Muscettola et al., 1984; Schatzberg et al., 1989; Ressler and Nemeroff, 1999; Strawn and Geraciotti, 2008), although the profile is far from clear. It has been suggested that variability in such measures may be related to the expression of different symptoms, or to different subtypes of depressive disorder (Gold and Chrousos, 1999).

There is more convincing evidence of catecholaminergic dysregulation and increased response sensitivity in PTSD (Strawn and Geraciotti, 2008). Elevated plasma NE levels have been correlated with greater symptom expression (Yehuda et al., 1992; Lemieux and Coe, 1995). Peripheral NE release in response to traumatic reminders is enhanced (Blanchard et al., 1991), and administration of yohimbine induces symptoms in PTSD patients (Southwick et al., 1993). Increased peripheral NE activity is consistent with the modestly elevated TH levels we observed in the adrenal medulla of stressed rats. By contrast, a limited study of post-mortem brain tissue from soldiers showed an approximate 50% reduction in the number of LC-NE neurons in the probable-PTSD group compared to controls (Bracha et al., 2005). A similar profile was seen in suicide victims, suggesting reduced NE signaling capacity in depression (Arango et al., 1996). These central changes are similar to the reduction of brainstem TH in PNS rats in the present study, and may thus be more indicative of reduced stress coping capacity and a predisposition to stress vulnerability than to the overt expression of stress-induced illness per se.

The HPA axis and glucocorticoids have also been implicated in affective disorders. In some depressed patients, there is evidence of elevated basal cortisol secretion (Board et al., 1956; Gold et al., 1986; Souetre et al., 1988; Arborelius et al., 1999), elevated corticotropin releasing hormone in cerebral spinal fluid (Nemeroff et al., 1984), blunted diurnal rhythms (Souetre et al., 1988), and impaired negative feedback by dexamethasone (reviewed in Handwerger, 2009).

Likewise, anxiety disorders are also associated with alterations in HPA axis activity, but there is considerable inconsistency in the literature. Of particular relevance to the present study, HPA dysregulation has been reported in PTSD, although the exact nature of the dysregulation remains a matter of debate. Some studies have shown elevated basal urinary and plasma cortisol levels in PTSD patients compared to controls (Hoffman et al., 1989; Pitman and Orr, 1990; Lemieux and Coe, 1995), while others have shown lower urinary and plasma cortisol (Mason et al., 1986; Yehuda et al., 1990, 1993, 1995; Boscarino, 1996). Again, discrepancies may be related to the nature or duration of the trauma, or to the expression of specific symptoms (de Quervain et al., 2009; Handwerger, 2009).

Sensitized fear and impaired extinction may be indicative of a more general cognitive deficit related specifically to hypoactivity in the mPFC. Noradrenergic signaling in the mPFC is implicated in tasks requiring cognitive flexibility (Lapiz and Morilak, 2006; Aston-Jones et al., 1999), and extinction learning is a form of cognitive flexibility that is dependent upon the functional integrity of the mPFC (Morgan et al., 1993). Cognitive dysfunction, including cognitive inflexibility and perseveration, is an important component of stress-related psychiatric disorders, and individuals with depression, obsessive-compulsive disorder, or PTSD perform poorly on tests of executive function and cognitive flexibility (Sutker et al., 1995; Fossati et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangarathnam and Asbjørnsen, 2007). Such cognitive deficits are often manifest in the form of negative biases, contributing to disordered thinking about self-worth, life stressors, and/or fear-provoking events (Coles and Heimberg, 2002; Elzinga and Bremner, 2002). It has been hypothesized that a hypoactive mPFC and associated cognitive deficits may not only contribute to the symptoms of affective disorders, but may also be a consequence of early-life stress exposure that creates a vulnerability to develop such disorders in response to later stress or trauma (Elzinga and Bremner, 2002).

The prenatal stress model of vulnerability shares some characteristics with genetic models of stress vulnerability, including Wistar-Kyoto (WKY) rats and high responder (HR) rats. WKY rats show a number of behavioral characteristics suggesting increased stress sensitivity, including heightened neophobia and depressive-like behaviors (Paré, 1994). Our laboratory has previously reported that WKY rats exhibit differences in both the expression and regulation of peripheral and central TH mRNA. Specifically, WKY rats showed attenuated TH induction and reduced NE release in the brain in response to acute stress under basal conditions, but greatly enhanced acute NE responses after chronic stress exposure, as well as increases in freezing behavior and acute HPA reactivity (Sands et al., 2000; Pardon et al., 2002, 2003; Ma and Morilak, 2004). Thus, noradrenergic dysregulation may contribute to stress-vulnerability in the WKY genetic model as well as that produced by PNS exposure in the present study. One difference is that prenatal stress induced basal changes in TH, whereas WKY rats did not differ in basal expression, but had attenuated acute stress-evoked induction of TH. In both models, the changes in TH expression may reflect reduced capacity for acute stress adaptation and coping, in-

cluding a preference for passive coping behaviors (i.e. freezing and/or immobility), which may be maladaptive in certain conditions.

HR rats are another rat model of stress vulnerability that share some neurobiological characteristics with the PNS-exposed rats, including elevated CORT, prolonged recovery of basal CORT levels post-stress (Piazza et al., 1991; Kabbaj et al., 2000), and decreased GR mRNA expression in hippocampus (Kabbaj et al., 2000). HR rats displayed increased locomotor responses to amphetamine (Piazza et al., 1989, 1991), as reported after PNS (Koenig et al., 2005), although we saw no changes in basal locomotion in the open field in the present study. However, HR rats have also been defined by high novelty-seeking behavior and enhanced exploration of anxiogenic environments (e.g. Kabbaj et al., 2000), whereas PNS rats have been reported to avoid such environments (Weinstock et al., 1992; Valle et al., 1997; Bosch et al., 2007; Fan et al., 2009). Thus, these models may produce different but overlapping behavioral and neurobiological manifestations that each may be informative for identifying mechanisms underlying individual differences in coping and stress vulnerability. The PNS/CAPS model exemplifies an experience-based stress vulnerability, which may help us to understand aspects of disorders such as PTSD, which present only after experiencing a severe stressor later in life.

Human psychopathologies are complex and multi-dimensional disorders, involving many brain systems and neural circuits. We would not suggest that PNS plus CAPS models all characteristics of any given affective disorder, nor does it replicate any human syndrome in its entirety in rats. Rather, it models key dimensions of many stress-related affective disorders, particularly those associated with fear and acute stress-reactivity. Moreover, it appears to model a vulnerability in specific neurobiological systems that can modulate the processes of fear conditioning and extinction. Thus, this model and others like it will allow a productive investigation of the potential mechanisms underlying long-term changes in the effectiveness of stress-coping capability, and of lifelong vulnerability to stress-induced psychopathology.

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